Cyclooxygenase-2–Dependent Prostacyclin Formation and Blood Pressure Homeostasis

Targeted Exchange of Cyclooxygenase Isoforms in Mice

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Rationale: Cyclooxygenase (COX)-derived prostanoids (PGs) are involved in blood pressure homeostasis. Both traditional nonsteroidal antiinflammatory drugs (NSAIDs) that inhibit COX-1 and COX-2 and NSAIDs designed to be selective for inhibition of COX-2 cause sodium retention and elevate blood pressure.

Objective: To elucidate the role of COX-2 in blood pressure homeostasis using COX-1>COX-2 mice, in which the COX-1 expression is controlled by COX-2 regulatory elements.

Methods and Results: COX-1>COX-2 mice developed systolic hypertension relative to wild types (WTs) on a high-salt diet (HSD); this was attenuated by a PGI_2 receptor agonist. HSD increased expression of COX-2 in WT mice and of COX-1 in COX-1>COX-2 mice in the inner renal medulla. The HSD augmented in all strains urinary prostanoid metabolite excretion, with the exception of the major PGI_2 metabolite that was suppressed on regular chow and unaltered by the HSD in both mutants. Furthermore, inner renal medullary expression of the receptor for PGI_2, but not for other prostanoids, was depressed by HSD in WT and even more so in both mutant strains. Increasing osmolarity augmented expression of COX-2 in WT renal medullary interstitial cells and again the increase in formation of PGI_2 observed in WTs was suppressed in cells derived from both mutants. Intramedullary infusion of the PGI_2 receptor agonist increased urine volume and sodium excretion in mice.

Conclusions: These studies suggest that dysregulated expression of the COX-2 dependent, PGI_2 biosynthesis/response pathway in the renal inner renal medulla undermines the homeostatic response to a HSD. Inhibition of this pathway may contribute directly to the hypertensive response to NSAIDs. (*Circ Res*. 2010;106:337-345.)

Key Words: cyclooxygenase-2 ■ nonsteroidal antiinflammatory drugs ■ hypertension ■ prostacyclin ■ PGI_2 receptor

Prostaglandins contribute to blood pressure (BP) homeostasis via their direct effects on vascular tone and on fluid and electrolyte transport in the kidney. Nonsteroidal antiinflammatory drugs (NSAIDs) block prostanoid (PG) biosynthesis by inhibition of the activity of the cyclooxygenase (COX) isozymes COX-1 and COX-2.[1] Both traditional (t)NSAIDs and those designed to be selective for inhibition of COX-2 may increase systemic BP and/or undermine BP control with antihypertensive drugs.[2,3] PG formation is generally reactive and elaboration of vasodilator PGs preserves renal blood flow in renoprival conditions.[4] Similarly, NSAIDs, in vulnerable populations, such as the elderly or in response to a hypertensive stimulus, such as a high-salt diet (HSD), decrease total renal perfusion and cause redistribution of renal blood flow.[5] This may lead to medullary ischemia, and even acute renal failure.[6] Even short-term studies of NSAIDs in apparently healthy, but susceptible, populations may result in decreased glomerular filtration rate and urinary sodium retention.[7]

In rodents, COX-1 deletion causes natriuresis, accentuates the effects of angiotensin converting enzyme inhibitors, and reduces BP despite activation of the renin–angiotensin system.[8] Indeed, both pharmacological inhibition and genetic deletion of COX-1 abolish the hypertensive response to angiotensin II in mice.[9,10] Deletion or inhibition of COX-2, by contrast, reduces renal medullary blood flow and sodium excretion, increases the vasoconstrictive response to angiotensin II,[10] and elevates basal BP.[11] These observations have prompted the suggestion that hypertension on NSAIDs is a function of both inhibition of COX-2 and the selectivity with which it is attained.[12] Although this is consistent with some evidence,[3,13] both the relative importance of selectivity and the mechanism by which COX-2 preserves BP homeostasis remain to be rigorously addressed.

Although the COX isozymes are structurally similar, their method of regulation is quite distinct.[1] One mechanism by which COX-2 might play a role in BP homeostasis is by its

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Non-standard Abbreviations and Acronyms	
BP	blood pressure
EC	endothelial cell
HSD	high-salt diet
IP	prostanoid I_2 receptor
KO	knockout
NSAID	nonsteroidal antiinflammatory drug
pdNSAID	NSAID purposefully developed to inhibit selectively COX-2
PG	prostanoid
RMIC	renal medullary interstitial cell
tNSAID	traditional NSAID
Tx	thromboxane
WT	wild type

ready induction in renal medullary interstitial cells (RMICs) where it is coexpressed with COX-1[14] and induced by high salt or fluid deprivation.[15,16] Inhibition of medullary COX-2 in rats[17,18] or global deletion of the PGI_2 receptor (IP)[19,20] or the EP_2 PGE_2 receptor[21,22] in mice results in salt-sensitive hypertension. We designed COX-1>COX-2 mice[23] to address the hypothesis that dysregulated expression of COX-2, rather than structural distinctions of COX-2[24] from COX-1, might have relevance to BP homeostasis. Our results support the notion that dysregulated expression of the COX-2 dependent, PGI_2 biosynthesis/response pathway in the renal internal medulla undermines the homeostatic response to a HSD. Inhibition of this pathway may contribute directly to the hypertensive response to NSAIDs.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Mice

All COX-1>COX-2 and COX-2 null mice used for the experiments were initially produced on a mixed C57BL/6 x Sv129 genetic background (50%:50%). All procedures were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

BP Measurement

Resting systolic blood was measured in conscious mice using a computerized noninvasive tail cuff system (Visitech Systems Inc) as previously described.[11]

Analysis of Renal Medullar Perfusion and Urinary Sodium Excretion

Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg IM), and the right kidney was removed. After 1 week of recovery, catheters were implanted in the jugular vein, bladder and renal medullary interstitium as described previously.[25] Saline or cicaprost tested compound was infused into the renal medulla at 20 μL/h. After 1 hour of equilibration, urine was collected every 30 minutes. Urinary sodium concentration was determined by Electrolyte Analyzer (PL 1000B).

Statistical Analysis

Data are presented as means±SEM. Analyses were performed by the ANOVA and subsequent pairwise comparisons as appropriate. A probability value of <0.05 was considered significant. Prism 4.0 software (GraphPad InStat 3) was used for all the calculations.

Results

COX-1 Rescues the BP and Renin Response to COX-2 Deletion on a Normal Diet but Not on a HSD

COX-2–deficient mice exhibited systolic hypertension (145.0±5.4 mm Hg versus wild-type [WT] littermates, 122.0±7.8 mm Hg; $P<0.01$; Figure 1A and Online Figure I), whereas BP was unaltered in COX-1>COX-2 mice BP (127.0±5.5 mm Hg versus WT littermates, 123.3±4.4 mm Hg; Figure 1A) on a normal chow diet. These results indicate COX-1 could substitute for COX-2 in maintaining BP homeostasis under physiological conditions. This is also consistent with the ability of COX-1 under the COX-2 promoter to rescue impaired renal development, characteristic of COX-2–null mice.[23,26] The renal cortical hypoplasia and biochemical evidence of renal dysfunction in COX-2 knockouts (KOs) was absent in COX-1>COX-2 mice (Online Figure II). However, in response to the HSD, COX-1>COX-2 mice developed hypertension (149.7±7.3 mm Hg versus WT, 128.1±6.5 mm Hg, $P<0.05$, n=9 to 12; Figure 1A), just like COX-2 KOs (151.0±2.9 mm Hg; $P<0.05$). Thus, although COX-1 could rescue the renal developmental impact of COX-2 deficiency, including hypertension on a chow diet, it was unable to compensate for the

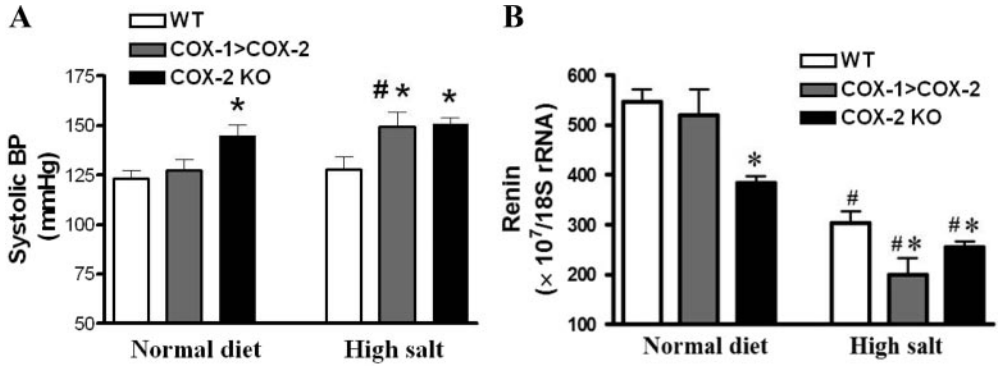

Figure 1. Effects of normal or HSD on BP and renin expression in COX-1>COX-2, COX-2 KO, and WT mice. A, Systolic BP was measured in conscious COX-1>COX-2, WT and COX-2 KO mice by tail cuff (n=9 to 12 for each group). Mice (6 to 7 weeks old) were fed either a normal chow diet (0.7% NaCl) or a HSD (8% NaCl) for 2 weeks. *$P<0.05$ vs WT controls; #$P<0.05$ vs normal diet. B, Quantitative real-time RT-PCR was performed to analyze renin mRNA expression in the kidneys from COX-1>COX-2, WT, and COX-2 KO mice. *$P<0.05$ vs WT controls; #$P<0.05$ vs normal diet (n=4 to 6).

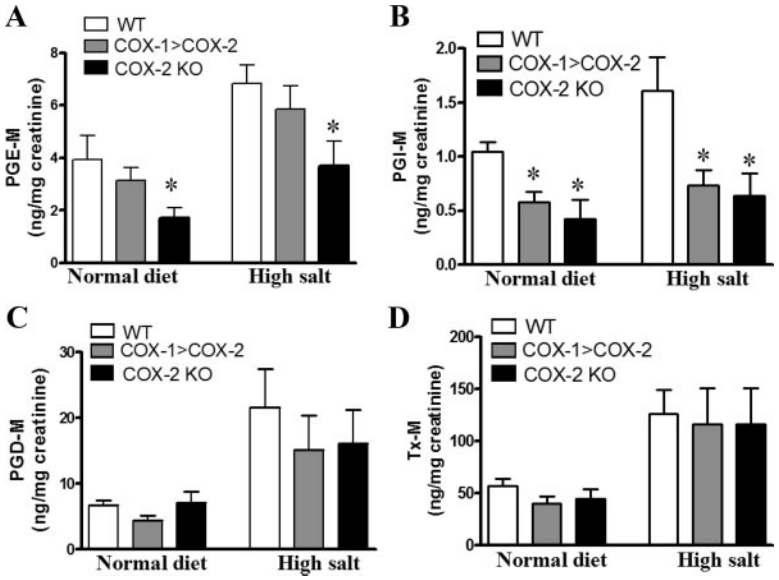

Figure 2. Effect of high salt intake on PG biosynthesis in COX-1>COX-2, COX-2 KO, and WT mice. Twenty-four-hour urine samples from COX-1>COX-2, COX-2 KO, and WT male mice (8 weeks old) were collected; the urinary PGE_2 metabolite (PGE-M) (A), PGI_2 metabolite (PGI-M) (B), PGD_2 metabolite (PGD-M) (C), Tx_2 metabolite (Tx-M) (D) were measured. Data are presented as means±SEMs. *$P<0.05$ vs WT controls (n=7 to 20).

absence of COX-2 in regulating BP homeostasis in the face of a high salt challenge.

Downstream products of COX-2 include renin secretagogues, like PGI_2.[27,28] Renin expression was reduced on a chow diet by ≈35% in COX-2 KO mice, and this phenotype was rescued in COX-1>COX-2 mice ($P<0.05$; Figure 1B). However, analogous to the BP response to HSD, the homeostatic decline in renin observed in COX-2 KOs was not rescued in the COX-1>COX-2 mice.

Insertion of COX-1 Does Not Substitute for COX-2–Derived Biosynthesis of PGI_2

We have previously shown in mice and humans that COX-2 is the dominant source of PGI_2 biosynthesis, as measured by its urinary metabolite, 2,3-dinor-6-keto $PGF_{1\alpha}$ (PGIM).[11,24] By contrast, the major urinary metabolite of PGE_2, 9,15-dioxo-11α-hydroxy-2,3,4,5-tetranor-prostane-1,20-dioic acid (PGEM), derives from both COX-1 and COX-2.[11] Deletion of COX-2 suppressed both urinary PGIM and PGEM in mice on a chow diet. However, although COX-1 insertion could rescue the decline in PGEM (1.72±0.38 ng/mg creatinine in COX-2 KO versus 3.15±0.47 ng/mg creatinine in COX-1>COX-2 versus 3.89±0.92 ng/mg creatinine in WT) (Figure 2A), it had no impact on the suppression of urinary PGIM (Figure 2B). A similar disparity in the ability of COX-1 to substitute for COX-2 applied under HSD conditions. HSD increased excretion of all metabolites consistent with induction of COX-2 in renal medulla (see below) in WT mice. Deletion of COX-2 had no impact on excretion of major metabolites of either PGD_2 (11,15-dioxo-9α-hydroxy-2,3,4,5-tetranorprostan-1,20-dioic acid) or thromboxane (Tx) (2,3-dinor-TxB_2), both of which derive predominantly from COX-1,[29,30] on either chow or HSD (Figure 2C and 2D).

Medullary COX-1 Expression in Response to HSD in the Absence of COX-2

HSD is recognized to have divergent effects on expression of COXs in kidney.[15] As expected, the HSD had no significant effect on WT COX-1 expression in both cortex and medulla (Figure 3A and 3C), whereas it downregulated COX-2 expression in cortex and upregulated COX-2 in medulla in WT mice (Figure 3B and 3D). Indeed, cortical expression of COX-1 tended to be higher in the mutants on regular chow and this change reached significance on a HSD. COX-2 expression was absent, as expected, in both cortex and medulla in the mutants (Figure 3B and 3D). By contrast, HSD upregulated expression of COX-1 in the cortex of COX-1>COX-2 ($760\pm30\times10^6$/18S rRNA, $P<0.05$) and COX-2 KO versus WT ($717\pm45\times10^6$/18S rRNA versus $503\pm34\times10^6$/18S rRNA, $P<0.05$) mice (Figure 3A) and of COX-1 in the medulla of COX-1>COX-2 ($3768\pm242\times10^6$/18S rRNA versus $2302\pm167\times10^6$/18S rRNA, $P<0.05$), whereas it did not regulate expression of medullary COX-1 when the isozyme was expressed under its own promoter (Figure 3C). Thus, COX-2 is usually the dominant isoform regulated by HSD in the medulla.

Medullary COX-1 Cannot Restore the COX-2–Dependent Capacity to Generate PGI_2

Given the relevance of COX-2 in the renal medulla to BP homeostasis,[17,18] the tissue capacity to generate PGs was assessed. This is quite distinct from estimation of systemic PG biosynthesis, as reflected by urinary metabolites; the capacity of tissues to generate PGs greatly exceeds actual synthetic rates in vivo.[31]

The most abundant product was PGE_2 (Figure 4). Both PGE_2 and PGI_2 (detected as 6-keto-$PGF_{1\alpha}$) are vasodilators and PGI_2 is also a potent renin secretagogue. Consistent with hypertension and hyporeninemia (Figure 1A and 1B), the capacity to generate these PGs is reduced in COX-2 KOs on a chow diet (Figure 4A and 4C). By contrast, the rather trivial medullary capacity to generate PGD_2 and $PGF_{2\alpha}$, both of which derive predominantly from COX-1, is retained (Figure 4B and 4D).

HSD augments the capacity to generate renal medullary PGs in WT mice. However, this capacity is attenuated in the case of PGI_2 in both mutant strains (Figure 4C). This indicates that COX-2 is the dominant source of this PG and it cannot be substituted for by COX-1 under these conditions. The HSD induced increase in PGE_2 is restrained only in COX-2 KOs,

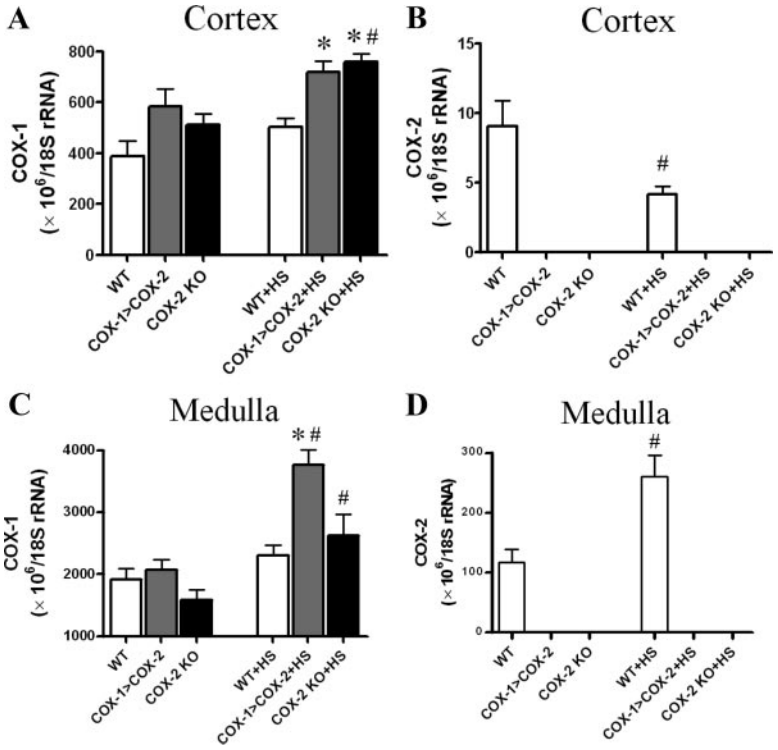

Figure 3. Effect of high salt intake on COX-1 and COX-2 expression in renal tissue obtained from COX-1>COX-2, COX-2 KO, and WT mice. COX-1 and COX-2 mRNA in renal cortex (A and B) and inner medulla (C and D) were determined by real-time RT-PCR. HS indicates HSD. *$P<0.05$ vs WT controls; #$P<0.05$ vs normal diet (n=4 to 6).

indicating the ability of COX-1 to compensate for COX-2 in the generation of this PG. Given the failure of COX-1 insertion to compensate for the BP effect of deletion of COX-2 (Figure 1), these results implicate PGI_2 as the dominant product of COX-2 restraining the hypertensive response to HSD.

Reduced Expression of the Medullary PGI_2 Receptor on a HSD

Although the EP1 and EP3 receptors for PGE_2 were, by far, the most abundantly expressed in renal medulla, their expression was unaltered in the mutants or by HSD. This was also true for the other receptors for PGE_2 (EP2 and EP4) and also for receptors for $PGF_{2\alpha}$ (the FPs) and for PGD_2 (the DP2). Expression of DP1 was not detectable (Online Figure III). Expression of the receptor for TxA_2, the TP, was suppressed compared to WT by COX-2 deletion on both chow and HSD (Online Figure III), although this was not evident in the renal cortex (Online Figure IV), where it is expressed in the glomeruli.[32] Pertinent to its proposed role in the hypertensive response to HSD, medullary expression of the PGI_2 receptor, the IP, is depressed by HSD, even in WT mice and expression of its transcript is detectably further depressed in both mutants (Figure 5A). Accordingly, we observed IP protein suppression by HSD was detectable in renal medulla, although no significant difference detected among genotypes (Figure 5B and 5C). Thus, decreased expression of the IP could interact with depressed synthesis of PGI_2 in the renal medulla to underlie the hypertensive response to COX-2

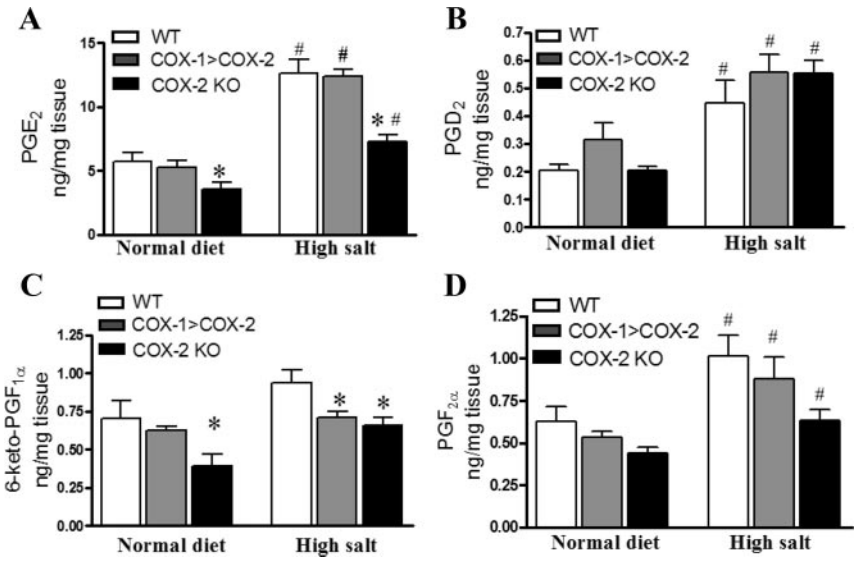

Figure 4. Effect of high salt intake on the capacity of renal medulla to generate PGs. Mice were fed a HSD or normal chow diet for 2 weeks, and the renal medulla was dissected and the PG profile (PGE_2 [A], PGD_2 [B], 6-keto-$PGF_{1\alpha}$ [C], $PGF_{2\alpha}$ [D]) was analyzed by mass spectrometry. *$P<0.05$ vs WT controls (n=5 to 8); #$P<0.05$ vs normal diet group (n=5 to 8).

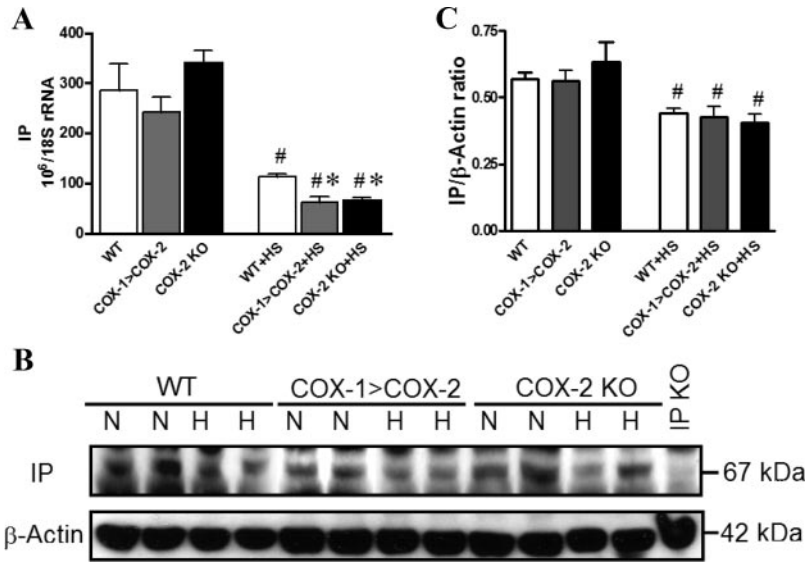

Figure 5. HSD decreases the medullary IP receptor expression in COX-1>COX-2, COX-2, and WT mice. A, IP mRNA levels in medulla from COX-1>COX-2, COX-2 KO, and WT mice were quantitated by real time RT-PCR. HS indicates HSD treatment. #$P<0.05$ vs normal diet; *$P<0.05$ vs WT controls (n=4 to 6). B, Representative Western blot of medullary IP receptor in COX-1>COX-2, COX-2 KO, and WT mice. N indicates normal salt diet; H, HSD. C, Relative level of IP protein to β-actin expression. #$P<0.05$ vs normal diet (n=4).

deletion and the failure of COX-1 to compensate for this deficiency. A similar pattern of decreased expression of the IP in both mutants on HSD was observed in the renal cortex (Online Figure IV), where it may be relevant to the attendant hyporeninemia (Figure 1B).

IP Receptor Activation Restrains Salt-Induced Hypertension in COX-1>COX-2 Mice

Systemic and renal PGI_2 biosynthesis were impaired under both basal and HSD conditions and renal IP receptor expression was further depressed by HSD in COX-1>COX-2 mice. To examine whether down-regulation of PGI_2/IP signaling contributes to salt-sensitive hypertension in COX-2 mutant mice, COX-1>COX-2 mice were subcutaneously infused with the IP specific agonist, cicaprost (50 μg/kg per day), using an osmotic minipump. BP was recorded before implantation and after exposure to a HSD by tail cuff. Systolic BP in COX-1>COX-2 mice was elevated markedly by the HSD and this effect was significantly attenuated by cicaprost infusion (from 140.8±4.1 to 119.4±4.2 mm Hg, $P<0.01$, n=9; Online Figure V).

Differential Substitution of COX-1 for Hypertonic Induction of COX-2–Dependent PG Formation in RMICs

In rodents, COX-2 expression is restricted in RMICs in renal medulla.[14] RMICs were isolated to address the impact of COX-1 substitution for COX-2 in an in vitro model of regulated expression of COX-2. The RMICs derived from COX-2 KOs were hypersensitive to cell dissociation, more than 45% of 3rd passage COX-2 KO RMICs died when treated with 0.025% trypsin/0.5 mmol/L EDTA compared to less than 3% in cells from either COX-1>COX-2 or WT groups. Unlike the cells from COX-1>COX-2 and WT mice (which passaged for more than 10 generations), even passage 4 RMICs were not derived successfully from COX-2 KO mice. The results are consistent with previous reports that COX-2 plays important role in RMIC survival.[16,33]

Consistent with our analysis of transcripts in the renal medulla of animals on a HSD (Figure 3), exposure of RMICs to hypertonic conditions (using addition of NaCl and mannitol to the culture medium) induced expression of COX-2 in WT cells and of COX-1 in those derived from COX-1>COX-2 mice (Figure 6A). The capacity of these cells to make PGs was augmented by 630 mOsm/kg H_2O (Figure 6B); again, PGE_2 is the most abundant product. Although the capacity to form PGI_2 is modest in these cells, induction of its formation is evident. Like PGE_2, deletion of COX-2 depresses significantly RMIC production of PGI_2. Substitution of COX-1 for COX-2 in the COX-1>COX-2 mice differentially rescued PG formation under hyperosmolar conditions. Thus, whereas 81%, 75%, and 60% capacity to form PGE_2, PGD_2, and $PGF_{2\alpha}$, respectively, was restored, COX-1 compensated only one-third of the capacity to form PGI_2 (measured as 6-keto-$PGF_{1\alpha}$).

Expression of the IP receptor in the renal medulla is largely restricted to the vasa recta.[34,35] Primary endothelial cells (ECs) and RMICs from same mice were grown in a transwell coculture system to address the possibility of paracrine signaling from RMICs via endothelial IPs. RMICs in the transwell insert were preincubated in media of high osmolality (630 mOsm/kg H_2O) to induce COX-2 expression, and intracellular cAMP (as a surrogate measure of IP activation) was quantified in ECs grown in the bottom chamber. Again, all PGs released from RMICs from WT in the coculture medium were augmented significantly by hypertonic stress. COX-1 replacement failed to recapitulate PGI_2 induction (measured as 6-keto-$PGF_{1\alpha}$) in RMICs from COX-1>COX-2 mice (Online Figure VI, A). Correspondingly, cAMP production of WT ECs was increased by 2.8-fold in response to stressed RMICs (from 3.6±0.27 to 9.8±1.48 pmol/10^5 cells, n=6; Online Figure VI, B), whereas only minimal cAMP in ECs from COX-1>COX-2 mice was detected (0.22±0.01 to 0.25±0.03 pmol/10^5 cells; Online Figure VI, B), and that was not increased by high osmolality. The cAMP response of ECs to cicaprost, did not differ between WT and mutant mice, demonstrating the capacity of the IP to generate the measured

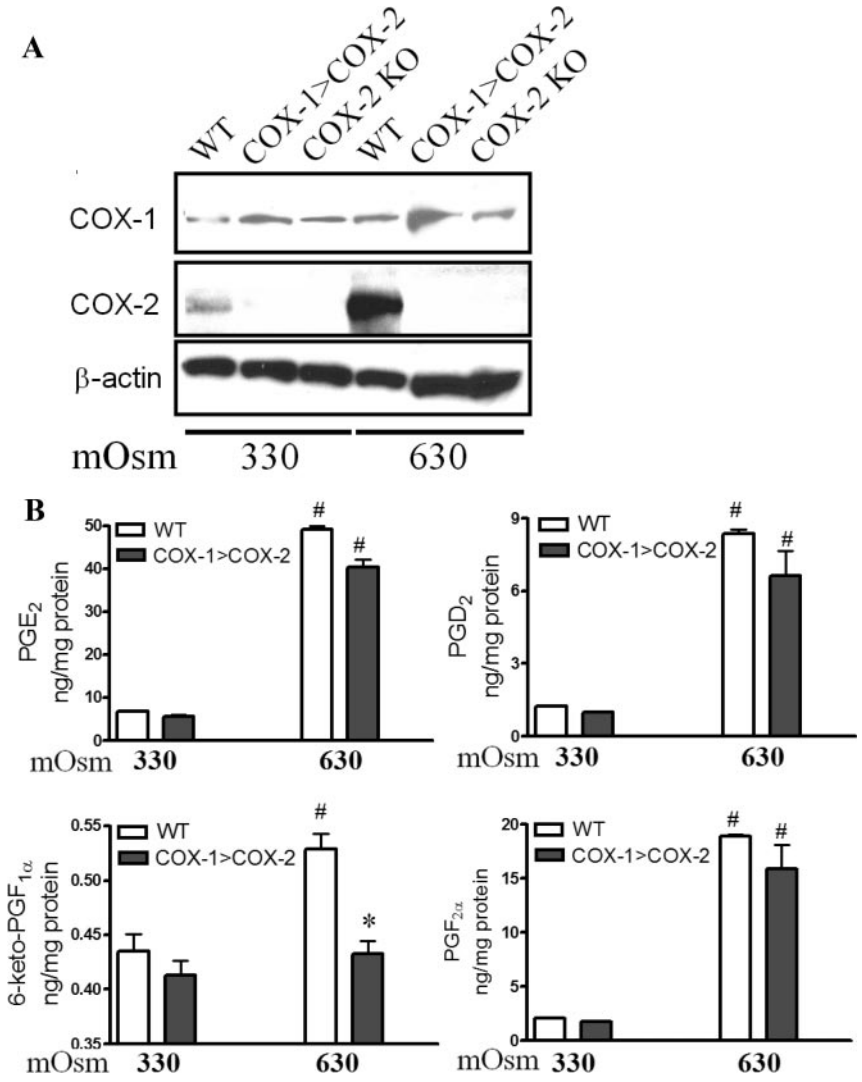

Figure 6. COX protein expression and PG production by cultured RMICs from COX-1>COX-2 and WT mice. RMICs grown to subconfluence were subjected to gradual changes in media osmolality from 330 to 630 mOsm/kg H_2O. After 24 hours, the cells were incubated with arachidonic acid (20 μmol/L) for 15 minutes. The cell lysates were prepared for Western blot of COX-1 and COX-2 (A), and the supernatants underwent PG analysis (B) by mass spectrometry. *$P<0.05$ vs WT; #$P<0.01$ vs 330 mOsm/kg H_2O group (n=6; repeated 3 times).

signal. These observations are consistent with aberrant IP receptor signaling in the vasa recta in COX-1>COX-2 mice.

Natriuresis Evoked by Activation of the Renal Medullary IP Receptor

The IP agonist cicaprost (10 ng/20 μL per hour) significantly and progressively increased urine volume from 0.69±0.09 μL/min (basal level, before infusion) to 1.67±0.10 μL/min ($P<0.01$; Figure 7A) and urinary sodium excretion from 0.095±0.002 μEq/min (basal level) to 0.1688±0.005 μEq/min ($P<0.01$; Figure 7B) over 2 hours of direct intramedullary infusion. In contrast, no significant effects on urine volume were observed over the same period during infusion of vehicle control (0.67±0.12 μL/min versus 0.74±0.05 μL/min, P=NS, n=4). Thus, activation of the renal medullary IP is capable of evoking natriuresis.

Discussion

Both tNSAIDs and NSAIDs purposefully developed to inhibit selectively COX-2 (pdNSAIDs) can elevate BP.[3] Vaso-

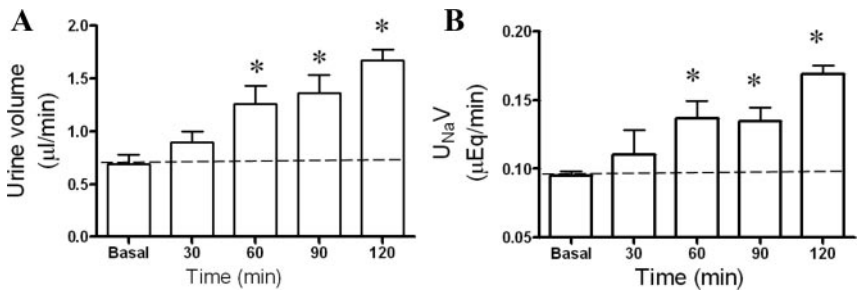

Figure 7. Activation of the IP by cicaprost evokes natriuresis in anesthetized mice. The right kidney was removed 1 week before the experiment. The mice were anesthetized and catheterized as described in Methods. After 1 hour of equilibration, cicaprost (10 ng/h) was infused into the renal medulla over 120 minutes. Urine was collected every 30 minutes before and during cicaprost infusion. Urine volume (UV) (A) and urinary sodium excretion ($U_{Na}V$) (B) were determined. Values presented as means±SEM. *$P<0.05$ vs basal level (n=6).

dilator PGs, such as PGI_2 and PGE_2, are critical to the preservation of renal blood flow in renoprival conditions[4,25] and COX-2 is the dominant source of biosynthesis of PGI_2, as assessed by excretion of its major urinary metabolite in humans and in mice.[11,36] Deletion of the IP results in salt-sensitive hypertension[19,20] and an increased sensitivity to thrombogenic stimuli,[11] whereas mutations of the IP and PGI_2 synthase have been associated with hypertension and cardiovascular events in humans.[37,38] Both COX-2 and COX-1 contribute to PGE_2 formation[11] and deletion of at least one of the EPs (the EP2) coupled, like the IP, to adenylate cyclase activation,[34] also results in salt-sensitive hypertension.[21,22] By contrast, activation of other PGE_2 receptors (EP1 and EP3) elevates BP[39,40] and activates platelets,[41] respectively, in rodents. Thus, PGI_2 and PGE_2 differ with respect to their relative derivation from COX-2 and their receptor dependent impact on cardiovascular function. Deletion and pharmacological inhibition of the COX isozymes suggests that their products have opposite effects on BP,[10] just as in hemostasis[3]; thus, the likelihood of a hypertensive response to any NSAID may relate both to inhibition of COX-2 and the selectivity with which it is attained.[12] Large scale clinical trials to address this hypothesis have not been performed. An overview analysis of 19 trials suggested that hypertension was more likely with pdNSAIDs than tNSAIDs. However, these trials were small and heterogeneous with respect to both groups of NSAIDs. Also, many tNSAIDs, such as diclofenac and meloxicam, are similarly selective to the pdNSAID celecoxib for inhibition of COX-2.[42]

The COX isozymes differ quite dramatically with respect to their transcriptional regulation.[1] These COX-1>COX-2 mice[23] permit assessment of whether harmonization of regulatory control allows rescue of COX-2 deficiency by COX-1. Our results suggest that COX-2 plays a unique role in maintaining BP homeostasis and that this relates to its preferential linkage to biosynthesis of renal medullary PGI_2.

The hypertensive response to NSAIDs is quite heterogeneous in humans[43] and is markedly influenced by genetic strain in mice.[44] The precise genetic modifiers of this response remain to be identified. Here we studied mice on a mixed C57BL/6 x Sv129 background, that exhibit hypertension after pharmacological inhibition or genetic deletion of COX-2 when maintained on regular chow.[11] COX-2 plays important roles in development[26] and mice deficient in COX-2 typically exhibit renal hypoplasia and biochemical evidence of renal compromise. Here, COX-1 can rescue the developmental effects, hypertension and hyporeninemia of COX-2 KOs under such physiological conditions. By contrast, COX-1 was unable to compensate for the impact of COX-2 deletion when the mice were exposed to the hypertensive stimulus of a HSD.

This response to a HSD reflects a selective impact on the medullary COX-2/PGI_2 biosynthetic/response pathway that mediates natriuresis. Evidence in the present study suggesting that COX-2 is uniquely coupled to synthesis of PGI_2 derives from metabolite measurement in vivo and estimates of the capacity to generate PGs in both kidney medulla and RMICs. Previous observations that PGI_2 was preferentially produced through COX-2 in macrophages,[45] ECs,[46] and COX/PGI synthase cotransfected HEK293 cells[47] are also consistent with this notion. COX-1 cannot compensate for the failure to augment PGI_2 formation in response to a HSD. This effect is likely amplified by the unexpected depression by the HSD of IP transcript and protein, uniquely among the PG receptors. Indeed, COX-2 deficiency in the mutant mice may have further augmented this effect, attaining significance at the level of IP mRNA.

Renal medullary COX-2 has previously been implicated in buffering the hypertensive response to dietary salt intake.[17,18] Both COX isoforms are abundant in medulla, but they are distributed differentially. COX-1 is expressed in both the medullary collecting duct and in RMICs.[10,14,15] COX-2 is most prominent in RMICs, which are involved in the maintenance of the medullary microcirculation and urinary salt excretion.[10] Although the renal papilla receives less than 1% of total renal blood flow, primarily through the descending vasa recta,[48] medullary blood flow promotes renal salt excretion, so called pressure natriuresis.[48]

Deletion of either the IP[19,20] or EP2 receptors[21,22] results in salt-sensitive hypertension, whereas deletion of the EP1[40] and $PGF_{2\alpha}$ receptor (FP)[49] reduces BP. IP receptors are expressed abundantly in the renal vasculature. IPs in the vasa recta[34,35] can indirectly modulate sodium reabsorption by altering blood flow to the adjacent medullary thick ascending limbs and collecting ducts.[50,51] Expression of the EP2 is also evident in the vasa recta,[52] where it too plays an important role in maintaining fluid and electrolyte balance and BP.[21,22,25] Indeed, PGE_2 is the most abundant PG product in the measurements of biosynthetic capacity in renal medulla and RMICs. However, unlike PGI_2, both systemic and renal medullary synthesis of PGE_2 can be rescued by COX-1 and rescue of PGE_2 is much more efficient than of PGI_2 in RMICs. Thus, PGI_2 is the COX-2 product more directly implicated in the preservation of BP homeostasis in response to dietary salt.

Placebo-controlled trials have revealed a cardiovascular hazard attributable to pdNSAIDs.[3] Although a predisposition to thrombosis is dominant, hypertension and cardiac failure are also evident in the spectrum of this cardiovascular risk.[53] Multiple lines of evidence suggest that the risk of thrombosis is attributable to suppression of COX-2 dependent PGI_2.[24,54] The present results integrate this mechanism with a predisposition to hypertension and provide in vivo evidence to support the notion that COX-2 is preferentially coupled to biosynthesis of PGI_2.

In summary, these results suggest that dysregulated expression of the COX-2–dependent, PGI_2 biosynthesis/response pathway in the renal internal medulla undermines the homeostatic response of BP to a HSD. Inhibition of this pathway may contribute directly to the hypertensive response to both pdNSAIDs and tNSAIDs in humans.

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Chair in Molecular, Cellular and Physiological Medicine and is recipient of a Career Investigator Award from the Heart and Stroke Foundation of Ontario.

Disclosures

G.A.F. is a consultant to Logical Therapeutics, receives support for an investigator initiated study from Crystal Genomics and serves of the scientific advisory board of Nicox; both of these companies have an interest in NSAIDs.

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Cyclooxygenase-2–Dependent Prostacyclin Formation and Blood Pressure Homeostasis: Targeted Exchange of Cyclooxygenase Isoforms in Mice

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Correction

Cyclooxygenase-2–Dependent Prostacyclin Formation and Blood Pressure Homeostasis: Targeted Exchange of Cyclooxygenase Isoforms in Mice: Correction

In the article that appears on page 337 of the February 5, 2010, issue, the name of one of the authors, Emer M. Smyth, was listed incorrectly. The full correct author list is reprinted below:

Ying Yu, Jane Stubbe, Salam Ibrahim, Wen-liang Song, Emer M. Smyth, Colin D. Funk, and Garret A. FitzGerald

The authors regret this error, and it has been noted in the online version of the article, which is available at http://circres.ahajournals.org/cgi/content/full/106/2/337

Reference

1.Yu Y, Stubbe J, Ibrahim S, Song W-l, Smyth EM, Funk CD, and FitzGerald GA. Cyclooxygenase-2–dependent prostacyclin formation and blood pressure homeostasis: targeted exchange of cyclooxygenase isoforms in mice. *Circ Res*. 2010;106:337–345.

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Supplement Meterial

Animal husbandry Animals were maintained on a 12-hour light/12-hour dark cycle with normal mouse chow (0.7% NaCl) and water provided *ad libitum*. As for high salt challenge, mice were fed with 8% NaCl chow diet for 2 weeks. All COX-1>COX-2 and COX-2 null mice used for the experiments were initially produced on a mixed C57BL/6 x Sv129 genetic background (50%:50%). Both genotypes had been individually maintained using a heterozygous by heterozygous cross breeding strategy for more than 20 generations.

The effect of Cicaprost on salt sensitive hypertension in mice 8-10 week old COX-1>COX-2 mice were randomly divided into two groups. One group was administered Cicaprost (50 μg/kg/d in 10 mM Tris/0.15 M NaCl solution, provided by Bayer Schering Pharma AG) subcutaneously via Alzet osmotic mini-pumps (model 2004) as described previously[1], another group received vehicle solution as a control (10 mM Tris/0.15 M NaCl solution). A HSD (8% NaCl) was initiated after implantation of osmotic mini-pumps and fed for an additional two weeks; blood pressure was recorded routinely by tail-cuff before mini-pump implantation and after HSD.

RMIC cell culture RMICs were isolated from male mice (10 weeks old) using a standard method described by Fontoura et al [2]. Medullary regions of kidney containing inner medulla and inner zone of the outer medulla were dissected. The dissected tissue was finely minced in Hanks' balanced salt solution (HBSS), then was digested with 170 unit/ml collagenase type I for 45 min at 37°C (Washington Biochemical). The digested tissue were further sieved through 105 um mesh before re-suspended in a 1:1 mixture of culture medium RPMI 1640 (20% fetal calf serum, 0.66 U/ml insulin, 2 g/l $NaHCO_3$, 15mM Hepes, 250 ug/l amphotericin B) and Dulbecco's modified Eagle's medium (DMEM: 10% fetal calf serum, 3.7 g/l $NaHCO_3$, 0.66 U/ml Insulin, 15mM Hepes, 250 ug/l amphotericin B) conditioned by 3T3 Swiss albino mouse fibroblasts in the log phase of growth. Cells were maintained at 37°C in 95% O_2-5% CO_2 incubator. These cells exhibited characteristic abundant oil red-O–positive lipid droplets. Cells were typically studied at their 3rd and 4th passages.

Culture medium osmolality could be modulated by adding extra NaCl and mannitol. The final concentration of NaCl and mannitol at 630 mOsm/kg.H_2O and 930 mOsm/kg H_2O

are 0.08 M and 0.1 M, 0.16 and 0.2, respectively. As for low osmolality of 230 mOsm/kg.H_2O, the culture medium was diluted 2:3 with distilled water. Once the desired osmolality was achieved, the cells were incubated for 24 h for further experiment.

Primary endothelial cell (EC) culture ECs were prepared from 6 week-old mice as previously discribed[3]. Briefly, lungs from two mice each group were harvested, minced finely and digested in collagenase (Worthington Biochemical Co, 200 U/ml) at 37°C for 45 minutes. After dissociated by titurating and filtered through a 100 μm disposable cell strainer (Becton Dickinson Labware), the lung cells were incubated with PECAM-1-coated beads (ratio: 1.5μg antibody to 10^7 beads) for 20 min at at 4°C, then recovered by magnetic separator and seeded in complete culture medium in 60 mm dish. When the cells reached 70 to 80% confluence (around 3 days), a 2nd sort by ICAM-2-coated beads was applied to get 99% EC purity[4]. Passage 3 cells were used for co-culture experiments.

RMIC/EC transwell co-culture and cellular cAMP Measurement 5×10^4 RMICs and 3×10^5 ECs each well were seeded in Transwell Insert (0.4 um pore, 12 Well, Corning Incorporated) and regular 12-well plate (Corning Incorporated), respectively. Indomethacin (10μM, Sigma) was included overnight in the EC medium to block endogenous PGs before co-culture. RMICs were changed to high osmolality medium (630 mOsm/kg.H_2O) or containing 5 μg/ml LPS for 5 hours to upregulate COX-2 expression, then placed over EC minelayers (1.5 ml/well serum-free medium containing 1 mM isobutylmethylxanthine (IBMX), phosphodiesterase inhibitior) and incubated for additional 2 hours. The serum-free medium was collected for PG analysis; cellular cAMP in cultured EC was extracted with ice-cold 65% ethanol for 30 min and then quantified using a radioimmunoassay kit (Amersham) according to the manufacturer's instructions.

Western blotting Cultured RMICs were washed with PBS and harvested in NuPAGE lysis buffer (invitrogen) followed by repetitive aspiration using a 27-gauge needle. Protein (10 μg) was loaded into each lane, separated on 4-10% BisTris-NuPAGE gels (Invitrogen) and transferred to Hybond ECL nitrocellulose membranes (Amersham Biosciences). Rabbit anti-COX-1 polyclonal antibody (Cayman Chemical Co.) at a 1:500 dilution, rabbit anti-COX-2 polyclonal antiserum (Cayman Chemical Co.) at a 1:1000 dilution, Rabbit anti-IP receptor polyclonal antibody (Cayman Chemical Co.) at a 1:500

dilution, and mouse anti-β-actin monoclonal antibody (Sigma) at a 1:5000 dilution were used as primary antibodies. Horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma) at 1:5000, and horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) at a 1:2000 dilution, were used as secondary antibodies, respectively. Signals were detected by ECL (Amersham Biosciences).

PG extraction and measurement Slices of inner medulla were carefully dissected and homogenized in 1 ml of ice cold PBS containing 100μM indomethacin using stainless steel beads (Qiagen). Residual tissue was separated by centrifugation, and the supernatant was collected. For PGs from cultured RMICs, cells were incubated with 30 μM arachidonic acid (AA, Cayman Chemical Co.) in PBS for 15 minutes. Either the supernatant from fresh tissues or culture medium was spiked immediately with 5ng PGD_2-d4 (Cayman Chemical Co, Cat# 312010), TxB_2-d4 (Cayman Chemical Co, Cat# 319030), 6-keto Prostaglandin $F_{1\alpha}$-d4 (Cayman Chemical Co, Cat# 315210), $PGF_{2\alpha}$-d4 (Cayman Chemical Co, Cat# 316010), PGE_2-d4 (Cayman Chemical Co, 314010), then purified by solid phase extraction using StrataX C18 cartridges (Phenomenex). The solid phase extraction cartridge was conditioned with 1 ml of acetonitrile and equilibrated with 1 ml of water. The sample was applied to the cartridge, which was then washed with 1 ml of 5% acetonitrile in water and dried with vacuum for 15 min. The analyte and internal standards were eluted from the cartridge using 1 ml of 5% acetonitrile in ethyl acetate. The eluate was collected and dried under a gentle stream of nitrogen. The resulting residue was reconstituted in 200 μl of 5% acetonitrile in water and filtered by centrifugation using 0.2-μm Nylon Microspin filters (Alltech Associates), then quantitated utilizing liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) analyses as described previously[5].

Urinary PG metabolite analysis 24-hour urines from male mice (8-10 weeks) were collected using metabolic cages and prostanoid metabolites were extracted and quantitated as previously described[6].

RNA isolation. Total RNA was extracted from tissues using RNeasy Mini-Kit (Qiagen). Reverse transcription was carried out on 400ng of RNA using Taqman Reverse transcription reagents (Applied Biosystems). The resulting cDNA was used for quantitative real time PCR.

Quantitative real time PCR TaqMan gene expression assays (Applied Biosystems, Foster City, Calif; catalog No. 4331182) for RENIN (Mm02342889_g1), COX-1 (Mm01336806_m1), COX-2 (Rn00568225_m1), EP1 (Mm00443098_g1), EP2 (Mm00436051_m1), EP3 (Mm00441045_m1), EP4 (Mm0043053_m1), FP (Mm00436055_m1), IP (Mm00801938_m1), TP (Mm00436917_m1), DP1 (Hs00235003_m1), DP2 (Rn00824628_m1) were performed on an ABI Prism 7900 Sequence Detection System. Results were normalized with 18S rRNA (Hs99999901_s1).

Histopathological Analysis Kidney and intestine were fixed in 10% buffered formalin for 24 h, processed routinely, and embedded in paraffin and stained with hematoxylin and eosin routinely.

Plasma BUN and creatinine Analysis Blood collected from the saphenous vein was analyzed for BUN levels by the clinic laboratory of the Veterinary Hospital of University of Pennsylvania.

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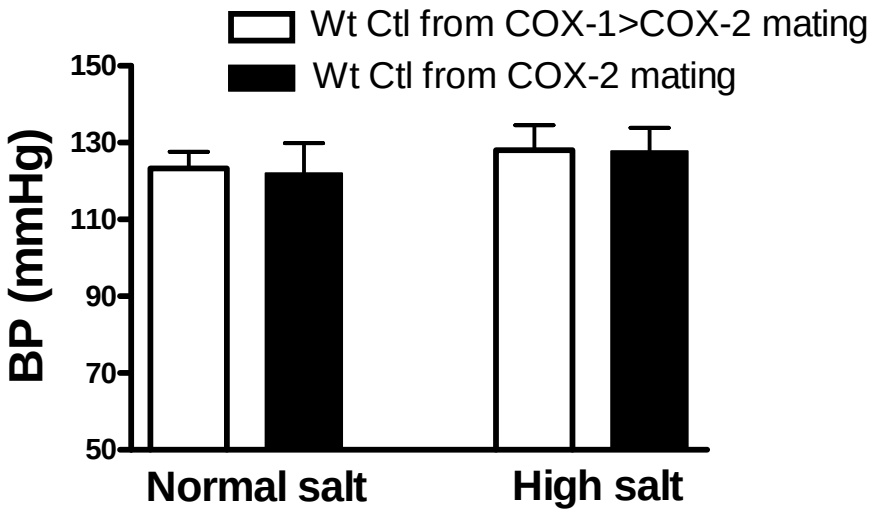
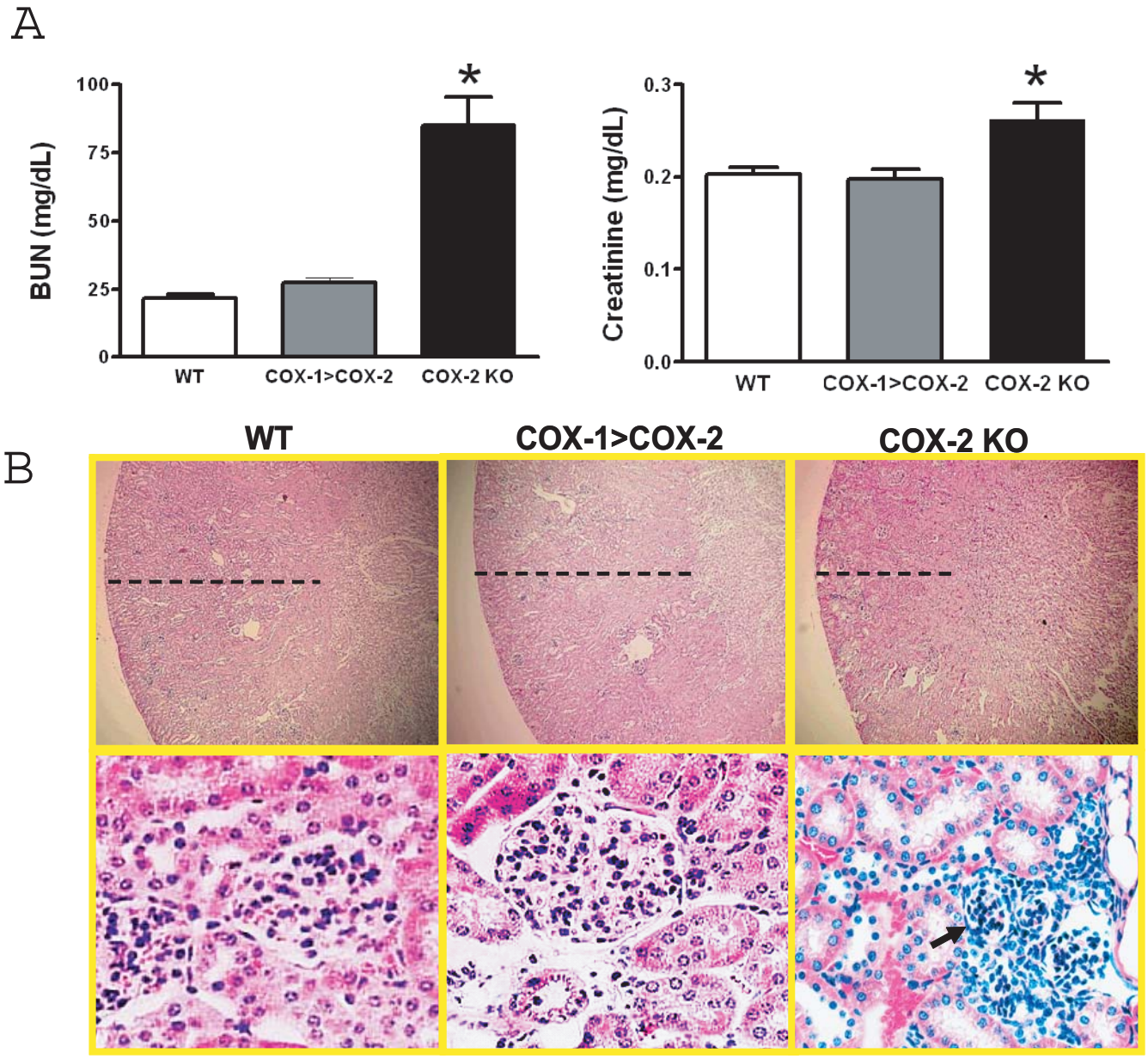
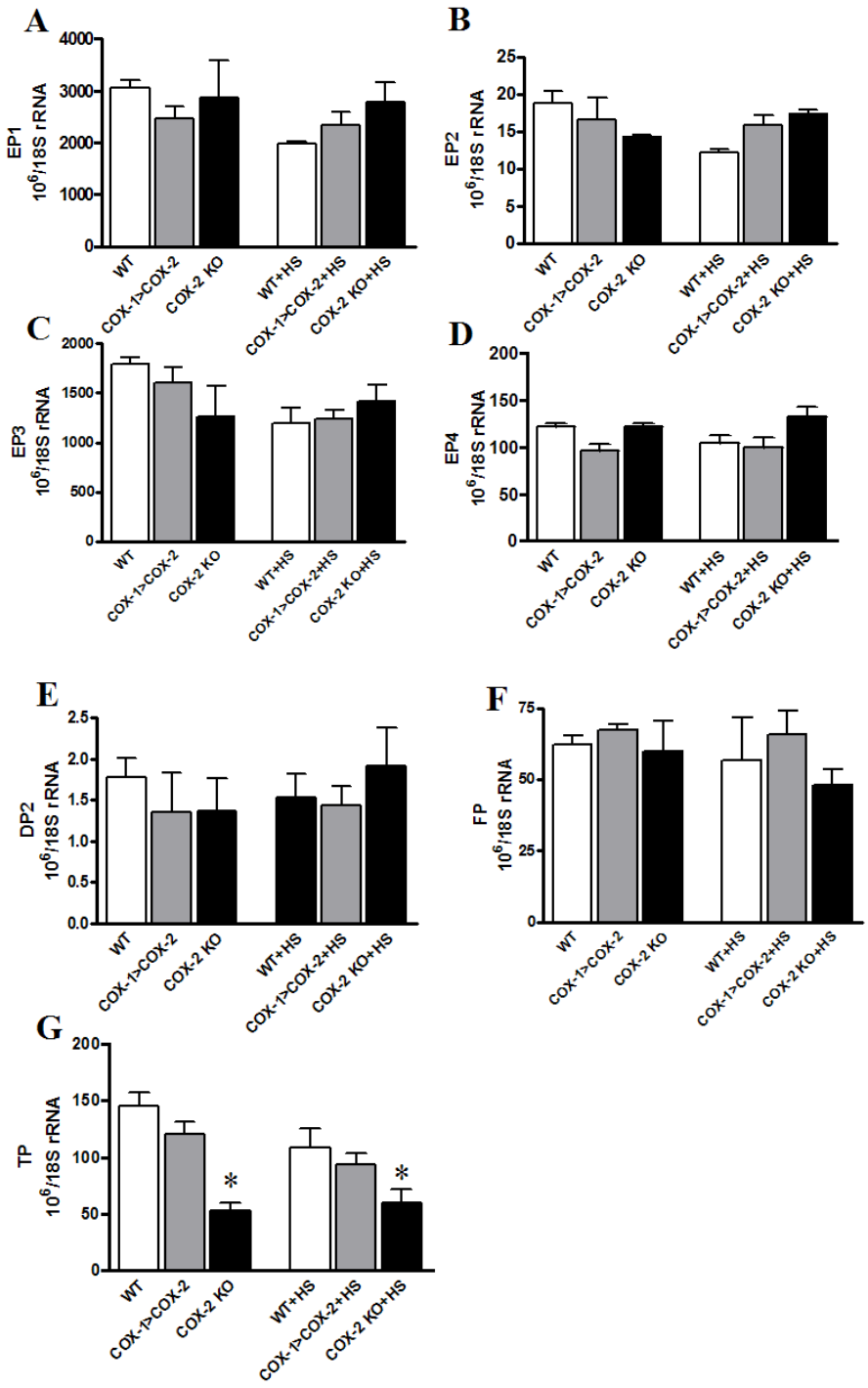


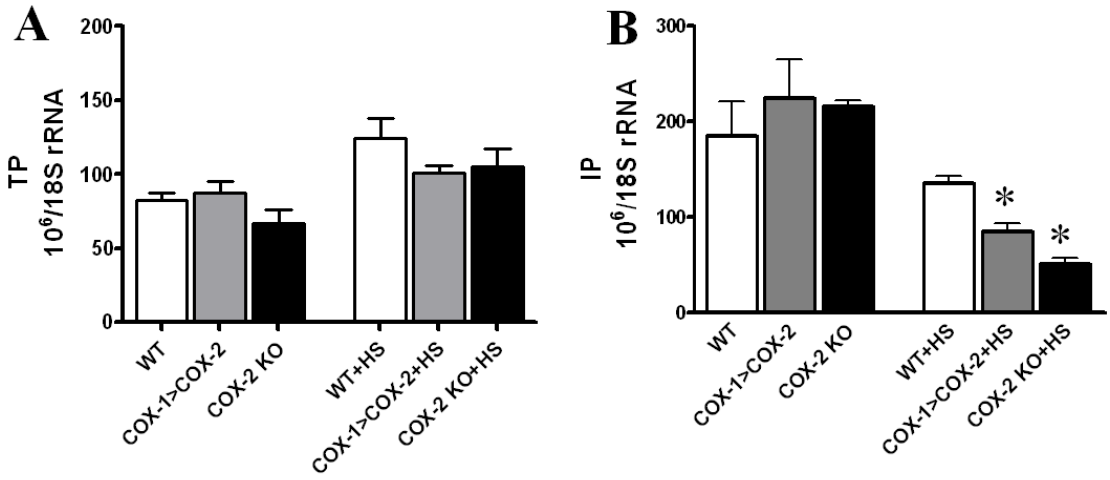
Online Figure I. **Comparison of blood pressure of WT mice from COX-1>COX-2 heterozygous mating and COX-2 heterozygous mating at normal chow diet and high salt diet.** Mice (6 weeks old) were fed either normal diet (0.7% NaCl) or high−salt diet (8% NaCl) for 2 weeks, blood pressure was measured by tail-cuff method. Wt Ctl, Wild type controls. N=10-11, p=n.s.



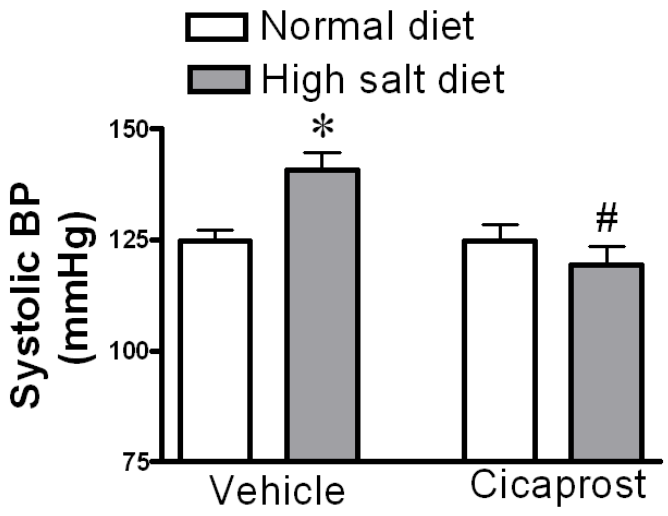
Online Figure II. Normal renal function in COX-1>COX-2 mice after high salt intake.
Mice (6-7 weeks old) were fed high salt diet (8% NaCl) for 4 weeks, plasma and kidney samples were collected for renal function analysis. A. Plasma blood urea nitrogen (BUN) and creatinine in WT, COX-1>COX-2 and COX-2 KO mice. *P<0.05 vs COX-1>COX-2 and WT mice, n=5-8.
B. Representative light photomicrographs of HE stained kidney sections from WT, COX-1>COX-2 and COX-2 mice after high salt treatment. Upper panel, 20X magnification; Lower panel, 400X magnification. Dotted lines represent the thickness of cortex. Black arrow, hypoplastic glomeruli near capsular surface .



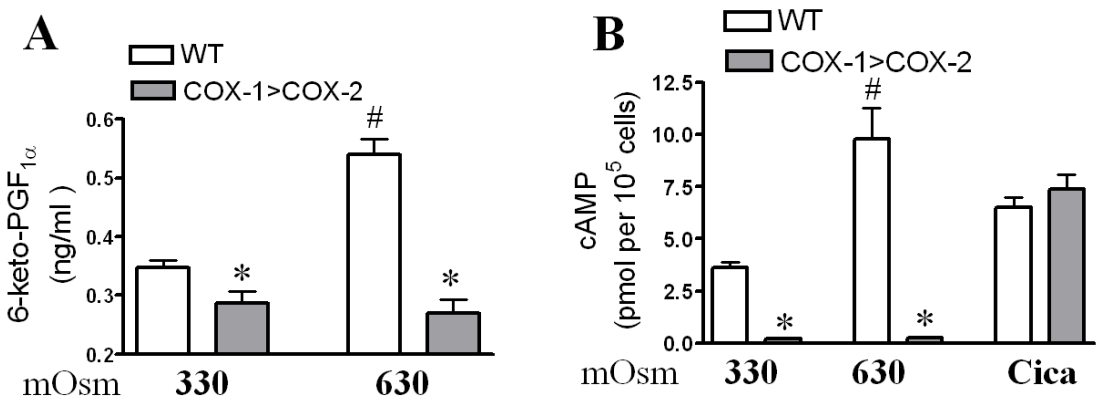
Online Figure III. Effect of high salt intake on PG receptor expression in inner medulla region in COX-1>COX-2, COX-2 and WT mice. EP receptors (**A, B, C, D**), DP2 (**E**), FP (**F**) and TP (**G**) mRNA level in medulla from COX-1>COX-2, COX-2 KO and WT mice were quantitated by real time RT-PCR; HS, High salt diet treatment. *, $p<0.05$ vs WT controls, n=4-6



Online Figure IV. TP (A) and IP (B) expression in renal cortex of COX-1>COX-2, COX-2 KO and WT mice before and after high salt treatment. TP and IP receptor in renal cortex from COX-1>COX-2, COX-2 and WT mice were quantitated by real time RT-PCR; HS, High salt diet treatment (2 wks). *, $p<0.05$ vs WT controls, n=4-6.



Online Figure V. The effect of Cicaprost on salt induced hypertension in COX-1>COX-2 mice. COX-1>COX-1 mice were infused subcutaneously either Cicaprost (50μg/kg/d in 10 mM Tris/0.15 M NaCl solution) or Vehicle (10 mM Tris/0.15 M NaCl solution) through Alzet osmotic mini pimps, and then subject to High salt diet (HSD) for 2 weeks. Blood pressure was recorded before implantation (Normal chow diet) and after HSD treatment. *, $p<0.01$ vs Normal diet; #, $p<0.01$ vs Vehicle group, n=9.



Online Figure VI. RMIC COX2 derived PGI_2/IP signaling was impaired in co-cultured vascular endothelial cells (ECs). RMICs in Transwell Inserts (Corning Incorporated) was sustained in either 330 or 630 mOsm/kg.H_2O culture medium for 5 hrs, then co-incubated with 1 mM isobutylmethylxanthine (IBMX) pretreated primary ECs for additional 2 hrs. The EC media were subjected to PG analysis (**A**) and cellular cAMP was assayed in cultured ECs (**B**). Cica, 1μM Cicaprost was used directly in IBMX-treated ECs medium as a positive control for IP activation. *, $p<0.05$ vs WT group; #, $p<0.01$ vs 330 mOsm group, n=6.